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Structure–activity relationships for the selectivity of hepatitis C virus NS3 protease inhibitors

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Abstract

The selectivity of hepatitis C virus (HCV) non-structural protein 3 (NS3) protease inhibitors was determined by evaluating their inhibitory effect on other serine proteases (human leukocyte elastase (HLE), porcine pancreatic elastase (PPE), bovine pancreatic chymotrypsin (BPC)) and a cysteine protease (cathepsin B). For these peptide inhibitors, the P1-side chain and the C-terminal group were the major determinants of selectivity. Inhibitors with electrophilic C-terminal residues were generally non-selective while compounds with non-electrophilic C-terminal residues were more selective. Furthermore, compounds with P1 aminobutyric acid residues were non-selective, while 1-aminocyclopropane-1-carboxylic acid (ACPC) and norvaline-based inhibitors were generally selective. The most potent and selective inhibitors of NS3 protease tested contained a non-electrophilic phenyl acyl sulfonamide C-terminal residue. HLE was most likely to be inhibited by the HCV protease inhibitors, in agreement with similar substrate specificities for these enzymes. The identified structure–activity relationships for selectivity are of significance for design of selective HCV NS3 protease inhibitors.

Keywords: Hepatitis C virus; NS3 protease; Inhibition; Selectivity; Specificity

1. Introduction

Since the discovery of hepatitis C virus (HCV) in 1989 by Choo et al. [1], a considerable effort has been made in search for an effective drug against HCV infections. About 2–3% of the earth's population is currently infected [2] and there is no specific etiological treatment available. HCV is a member of the *Flaviviridae* family whose non-structural protein 3 (NS3) comprises a bifunctional protease/helicase. The protease domain is a chymotrypsin-like serine protease, responsible for the cleavage between most of the non-structural proteins in the single polyprotein of HCV [3,4]. Recent results have confirmed the vital role of NS3 protease in the viral life cycle [5,6], validating the protease activity of the NS3 protein as a major target for anti-HCV drug development. Many types of HCV protease inhibitors have emerged [7,8], and it is important to identify structural characteristics that correlate with clinical efficacy. Clinical data for an HCV protease inhibitor (BILN 2061) has just been released [9], an important first "proof-of-concept". But various in vitro model systems still have to be used to identify inhibitors with high drug potential.

Our previous studies have resulted in a series of potent peptide-based protease inhibitors of full-length HCV NS3 [10–12]. Before performing further optimization towards higher inhibitory potencies and better pharmacokinetic properties, it was of importance to evaluate the selectivity of the inhibitors. The substrate specificity of proteases is generally defined by the side chains flanking the cleavage site [13]. The naturally occurring residue in the P1-position of HCV protease substrates, cysteine, is unusual in this position, providing a unique feature of potential importance for selectivity. However, thiol groups are generally not

Abbreviations: ACPC, 1-aminocyclopropane-1-carboxylic acid; Abu, L-aminobutyric acid; BPC, bovine pancreatic chymotrypsin; HCV, hepatitis C virus; HLE, human leukocyte elastase; NS3, non-structural protein 3; Nva, L-norvaline; PPE, porcine pancreatic elastase; SI, selectivity index

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suitable in drugs and we have therefore tested various isosteric replacements; one of the aims of this study was to compare the selectivity of these residues. Furthermore, the importance of the C-terminal group for the selectivity of these inhibitors was also of interest; especially the differences in the selectivity of compounds with two different C-terminal groups: electrophilic and non-electrophilic. Electrophilic compounds are well-known inhibitors of serine [14–18] and cysteine [19–21] proteases, and have also been found to be efficient inhibitors of the HCV NS3 protease [22,23]. Inhibition by electrophilic compounds is a result of a reaction between the electrophile and the catalytic serine or cysteine in the active site of the enzyme, leading to the formation of a covalent bond (Fig. 1a). Non-electrophilic inhibitors, on the other hand, rely on several non-covalent interactions with the substrate-binding site of a protease (Fig. 1b). The mechanisms and determinants for high efficacy are thus quite different for these two types of inhibitors.

Reference enzymes that were structurally and/or mechanistically similar to the target enzyme were chosen for the selectivity studies. They comprised three members of the chymotrypsin family of serine proteases: human leukocyte elastase (HLE), porcine pancreatic elastase (PPE), bovine pancreatic chymotrypsin (BPC) and a cysteine protease: human liver cathepsin B (CatB). HLE (also known as human neutrophil elastase) was of interest since it is a human enzyme with many important physiological functions, although it is also implicated in many pathological states (for an overview, see Ref. [24]). It has similar substrate specificity as HCV protease, having high activity with substrates containing Val in the P1-position, but also with Ala, Ser or Cys in P1. PPE is a structurally related enzyme with slightly different substrate specificity, accepting Gly in addition to Ala, Ser or Val, in the P1-position, but not Cys [25]. BPC is also a structurally similar serine protease, but with a completely different substrate specificity, preferring large hydrophobic residues; Tyr, Phe, Trp or Leu as P1-residues [26]. Although these pancreatic enzymes (PPE and BPC) were not of human origin, there are corresponding pancreatic proteases of importance for digestion in humans. Finally, cathepsin B is a cysteine protease with a similar catalytic mechanism as the serine proteases, but a different catalytic nucleophile and overall structure. It is specific for substrates with large hydrophobic residues or Arg as P2-residues [27]. These characteristics of the reference enzymes allowed fundamental structural aspects of the selectivity of the HCV inhibitors to be addressed.

2. Materials and methods

2.1. Inhibitors

The synthesis of the HCV NS3 protease inhibitors used in this study has been published previously: compounds 1–

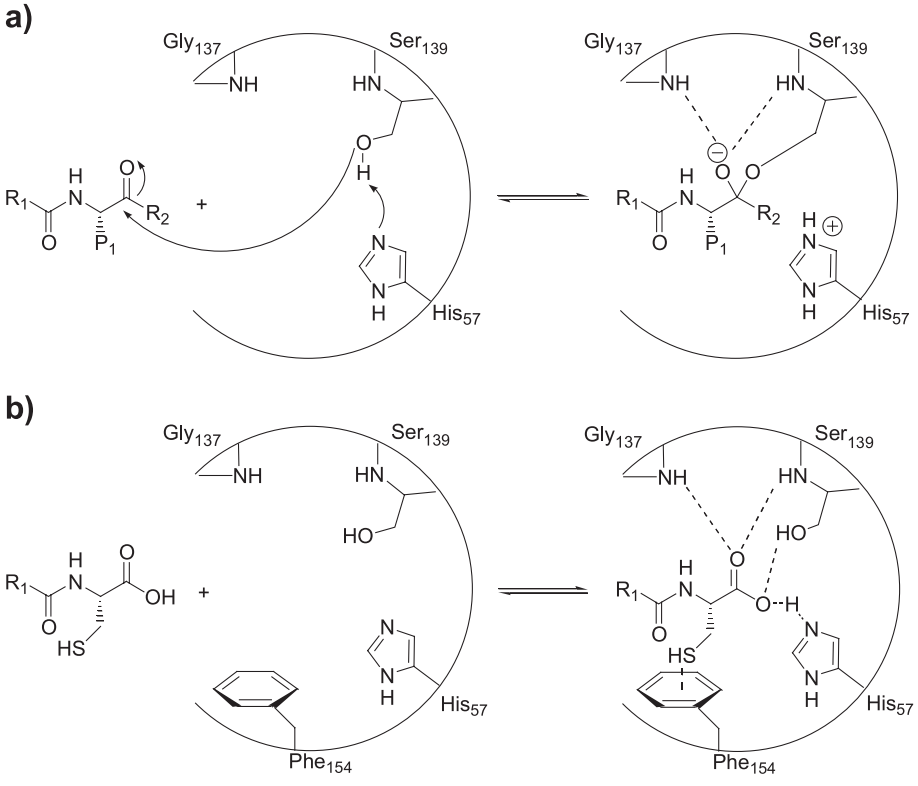


Fig. 1. Illustration of the assumed interaction of electrophilic (a) and non-electrophilic (b) inhibitors with HCV NS3 protease.

5, 15–18, and 20–23 in Ref. [10], compounds 6–14 and 19 in Ref. [12]. All inhibitors were dissolved in DMSO and stored at −20 °C between measurements.

2.2. Enzymatic assays

Reference enzymes; HLE, PPE type I, BPC, cathepsin B from human liver and their substrates were purchased from Calbiochem (La Jolla, CA, USA). The enzymatic activities of HLE, PPE, BPC and cathepsin B were determined by continuous measurements of the absorption of *p*-nitroaniline, released upon substrate cleavage ($\lambda = 385$ nm). Measurements were performed with a SPECTRAmax Microplate Spectrophotometer (Molecular Devices, Sunnyvale, USA). The activities were followed for 1 h to detect time-dependent enzyme inactivation, slow-binding inhibition and possible irreversible inhibition.

GraFit (Erithacus Software, Staines, MX, UK) was used for non-linear regression analysis of the data. K_m and V_{max} values were determined by non-linear fits of the Michaelis–Menten equation to initial reaction rate velocities (v_0) at different substrate concentrations.

HLE was dissolved in 50 mM Na acetate, pH 5.5 and 200 mM NaCl to 100 μg/ml and stored at −80 °C. Elastase substrate I (MeOSuc-Ala-Ala-Pro-Val-pNA) was dissolved in DMSO to a 10 mM final concentration. The enzyme was pre-incubated for 10 min at 30 °C in 50 mM Tris, pH 7.4 and 100 mM NaCl; the reaction was started by addition of the substrate. The final DMSO concentration was 3.25%. The K_m value was determined using 8–125 μM substrate and 17 nM enzyme. Inhibition measurements were performed with 125 μM substrate and 17 nM enzyme. Inhibitor (or DMSO in a blank reaction) was added to the reaction mixture immediately after the substrate.

PPE was dissolved in 50 mM Tris, pH 8.0 to 1 mg/ml and stored at −20 °C. Elastase substrate IV (Suc-Ala-Ala-Pro-Abu-pNA) was dissolved in a 1:1 mixture of H_2O and DMSO to a 5 mM final concentration. The substrate was pre-incubated for 10 min at 30 °C in 50 mM Tris, pH 8.0 and the reaction was started by addition of the enzyme. The final DMSO concentration was 5.75%. The K_m value was determined using 8–125 μM substrate and 1–20 nM enzyme. Inhibition measurements were performed with 125 μM substrate and 1 nM enzyme. Inhibitor (or DMSO in a blank reaction) was pre-incubated with the substrate and the reaction was started by addition of the enzyme.

BPC was dissolved in 50 mM Tris, pH 8.0 and stored at −20 °C. The chymotrypsin substrate (Suc-Gly-Gly-Phe-pNA) was dissolved in a 1:1 mixture of H_2O and DMSO to a 10 mM final concentration. The substrate was pre-incubated at 30 °C for 10 min in 50 mM Tris, pH 8.0 and the reaction was started by addition of the enzyme. The final DMSO concentration was 3.25%. The K_m value was determined using 0.125–1 mM substrate and 20 nM enzyme. Inhibition measurements were performed with 500 μM substrate and 20 nM enzyme. Inhibitor (or DMSO in a blank reaction) was pre-incubated with the assay mixture prior to addition of the enzyme.

A stock solution of cathepsin B was stored at −80 °C and diluted in 50 mM sodium acetate, pH 5.5, 2 mM DTT and 2 mM EDTA before use. Cathepsin B substrate I (Z-Arg-Arg-pNA) was dissolved in H_2O to a 5 mM final concentration. The substrate was pre-incubated at 30 °C for 10 min in 50 mM sodium acetate, pH 5.5, 2 mM DTT and 2 mM EDTA, and the reaction was started by addition of the enzyme. The final DMSO concentration was 3.25%. The K_m value was determined using 62–250 mM substrate and 100 mU/ml enzyme. Inhibition measurements were performed with 250 μM substrate and 53 μU/ml enzyme. Inhibitor (or DMSO in a blank reaction) was pre-incubated in the assay mixture prior to addition of the enzyme.

The expression, purification and inhibition studies of the full-length NS3 were described previously [28]. The assay was slightly modified for detection of the slow-binding inhibition. Briefly, 2 μM substrate and 25 μM cofactor were pre-incubated at 30 °C for 10 min in 50 mM HEPES pH 7.5, 10 mM DTT, 40% glycerol, 0.1% *n*-octyl-β-D-glucoside and 1.7% DMSO. The reaction was started by addition of 1 nM enzyme, and 5 μl of inhibitor (or DMSO in a blank reaction) was added 45 s later. The final DMSO concentration was 3.3%. The K_m value was determined as previously [28].

2.3. Inhibition measurements

All compounds were initially tested at a high concentration (100–200 μM) to get an estimate of their inhibitory effect, expressed as the percentage of inhibition. The progress curve was followed for 1 h or until the substrate was substantially depleted or enzyme inactivation was obvious (HLE, BPC). K_i values were only determined for compounds that caused more than 50% of enzyme inhibition at the highest concentration initially used. The risk of obtaining erroneous results at this concentration is high due to possible solubility problems [11]; besides, most of the inhibitors have K_i much lower than 200 μM with NS3 protease.

Initial rates were calculated from the slope of the linear part of the progress curves when inhibitor binding was fast. K_i values were calculated from initial velocities at different inhibitor concentrations by non-linear regression analysis using the standard equation for competitive inhibition [29] and K_m values determined separately since inhibition was measured at a single substrate concentration.

The progress curves of the inhibited reactions were analyzed for slow binding inhibition [30]. In cases where slow-binding inhibition was observed, the steady state velocity was calculated from the linear steady state part of the progress curve. This steady state reaction rate was treated as (v_0) and was used for K_i calculation as for fast binding inhibitors. Pipetting and mixing of solutions prior to the start of the reaction took on average 30–40 s, so only inhibitors that did not reach steady state by that time could be observed as being slow-binding.

2.4. Selectivity index

The selectivity index (SI) was calculated according to Eq. (1):

$$\mathrm{SI} = \frac{K_{ip}}{K_i} \quad (1)$$

Where K_i is the inhibition constant of the inhibitor for NS3 protease and K_{ip} is the inhibition constant of the inhibitor for HLE, PPE, BPC or cathepsin B. The SI of inhibitors, which produced less than 50% inhibition at the highest concentration, were estimated using an approximate inhibition constant, calculated from the IC_{50} value according to Eq. (2):

$$K_i = \frac{\mathrm{IC}_{50}}{I + \frac{[S]}{K_\mathrm{m}}} \quad (2)$$

The IC_{50} value was estimated according to Eq. (3):

$$\mathrm{IC}_{50} = \left(\frac{1}{\left(1 - \frac{v_i}{v_o}\right)} - 1 \right) \times [I] \quad (3)$$

where v_0 is the rate of the reaction in the absence of inhibitor and v_i is the rate of the reaction in the presence of inhibitor at a concentration [I] [29].

3. Results

A series of hexapeptide HCV NS3 protease inhibitors with variations in the structure of the N-terminal capping group (R_1), the P1 side chain and the C-terminal group (R_2), but identical core sequences (R_1-Asp-DGlu-Leu-Ile-Cha-R_2) were selected for inhibition studies with HLE, PPE, BPC and cathepsin B (Table 1). The N-terminal capping group was either acetyl (Ac) or succinic acid (Suc), while the P1-side chain was that of cysteine, L-aminobutyric acid (Abu), 1-aminocyclopropane-1-carboxylic acid (ACPC) or L-norvaline (Nva). Compounds **1**–**11** had electrophilic C-terminal residues, while compounds **12**–**23** were non-electrophilic.

3.1. Inhibition analysis

K_i values for the inhibition of HCV NS3 protease were taken from previous publications [10,12]. In this study, K_i values for the reference proteases were determined when more than 50% inhibition could be measured with 100 or 200 μM inhibitor; otherwise, the degree of inhibition (%) was determined at one of these inhibitor concentrations (Table 1). In order to obtain comparable data with as many of the compounds and enzymes as possible, suitable buffer conditions, enzyme and substrate concentrations, and time frames for inhibition measurements with each of the proteases and their corresponding substrates were established. The choice of substrate concentration was defined by the K_m values under the present conditions (56 μM for HLE, 29 μM for PPE, 1000 μM for BPC and 321 μM for cathepsin B). In order to avoid artifactual inhibition data, progress curves were carefully analyzed for indications of slow-binding or irreversible inhibition. HLE and BPC were found to lose activity during the course of the experiment, even in the absence of inhibitor. In some cases, the addition of inhibitor stabilized the enzymes. However, the part of the progress curve used for analysis was minimized and inhibition analysis was only precluded for two compounds (**6** and **12**).

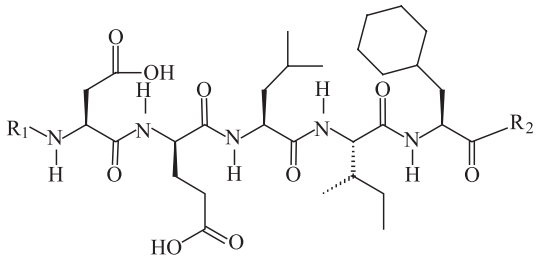
None of the compounds exhibited slow-binding inhibition of NS3 protease or irreversible inhibition with any of the enzymes. However, eight of the eleven electrophilic compounds were slow-binding with at least one of the reference proteases (Table 1, labelled [c]); none of the non-electrophilic inhibitors displayed slow-binding inhibition. All reference proteases showed evidence of this type of inhibition by at least one compound. Furthermore, slow-binding inhibition was only observed for compounds with high inhibitory potency; for example, inhibition of cathepsin B by the electrophilic compounds was generally slow binding, unless the inhibition was weak. A similar correlation between inhibitory effect and detection of slow-binding inhibition also existed for PPE and BPC. In contrast, HLE only showed this phenomenon for one inhibitor (compound **5**), despite more effective inhibition by many of the other compounds.

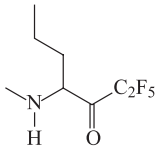
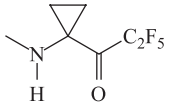
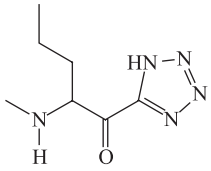
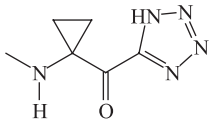
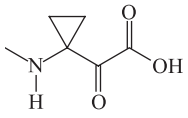
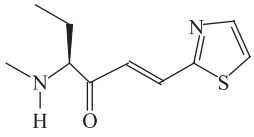
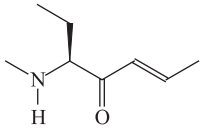
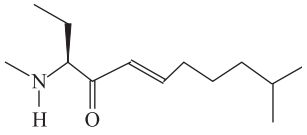
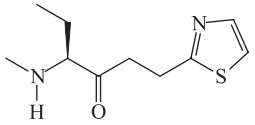
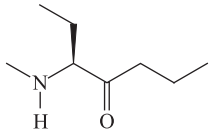
3.2. Inhibitor selectivity

In addition to inhibition data, the SI was estimated when possible (Table 1). Since it describes the inhibition of the reference enzyme relative that of the HCV protease, it facilitates comparisons between compounds with different inhibitory potencies on HCV protease. High SI values indicate high selectivity for HCV protease! Obviously, this measure is only a rough estimate when K_i values cannot be determined directly, and it does not exist when there is no inhibition (marked n.i. in the table). However, the analysis gives a useful estimate of selectivity even when accurate values cannot be determined.

The electrophilic compounds (**1**–**11**) were generally not selective for NS3 protease. The Nva-based pentafluoroethylketone and α-ketotetrazole inhibitors (**1** and **3**, respectively) inhibited all reference serine proteases, while their ACPC-based counterparts (**2** and **4**) inhibited HLE and BPC. The Nva α-ketotetrazole compound (**3**) was different to the other Nva and α-ketotetrazole compounds (**1** and **4**, respectively), being an effective inhibitor of HLE, PPE and cathepsin B. Obviously, selectivity cannot be predicted by combining data from different inhibitors. The potent ACPC-based α-keto acid inhibitor of NS3 protease (**5**), inhibited HLE and cathepsin B while the electrophilic Abu-based

Table 1
Inhibition data and selectivity index for HCV NS3 protease inhibitors with HLE, PPE, BPC and cathepsin B



Comp.	R_1	R_2	K_i (μM), or inhibition (%)					Selectivity index (SI)			
			NS3	HLE	PPE	BPC	CatB	HLE	PPE	BPC	CatB
1	Suc		0.12[a]	0.35 ± 0.2	0.065 ± 0.0013[c]	4.8 ± 1.1[c]	0% at 100 μM	2.8	0.5	38	n.i.
2	Suc		1.8[a]	5.6 ± 1.5	0% at 100 μM	21% at 100 μM	0% at 100 μM	3	n.i.	140[d]	n.i.
3	Suc		0.30[a]	8.1 ± 1.8	7.9 ± 1.7[c]	15% at 100 μM	45 ± 8	27	26	1300[d]	150
4	Suc		1.9[a]	45% at 100 μM	0% at 100 μM	8% at 100 μM	0% at 100 μM	20[d]	n.i.	410[d]	n.i.
5	Suc		0.098[a]	49% at 100 μM[c]	0% at 100 μM	3% at 100 μM	19 ± 2[c]	330[d]	n.i.	22000[d]	194
6	Ac		0.32[b]	n.d.	4% at 100 μM	8.6 ± 2.4[c]	4.7 ± 0.5[c]	n.d.	1400[d]	25	15
7	Ac		0.20[b]	16.2 + 1.4	29% at 100 μM	35 ± 12[c]	9.1 ± 0.7[c]	81	230[d]	165	45
8	Ac		0.39[b]	0.44 ± 0.04	0% at 100 μM	32 ± 7.7	1.54 ± 0.11[c]	1.1	n.i.	82	4
9	Ac		≈1	3.1 ± 0.2	9% at 100 μM	38 ± 5	9.3 ± 0.8[c]	3.1	190[d]	45	9
10	Ac		0.65[b]	1.77 ± 0.18	33% at 100 μM	18 ± 3[c]	30% at 100 μM	2.7	60[d]	29	200[d]

(continued on next page)

Table 1 (*continued*)

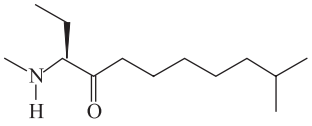
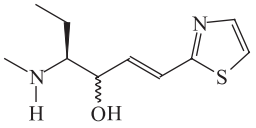
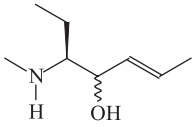
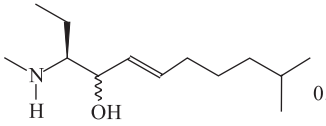
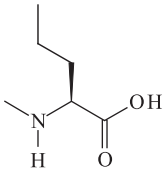
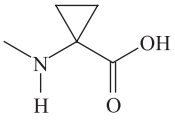
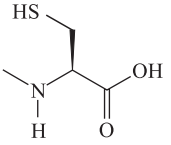
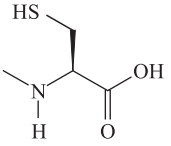
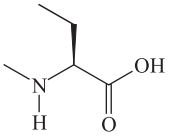
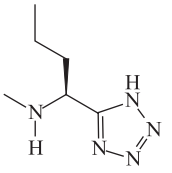
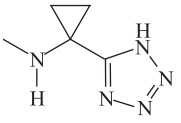
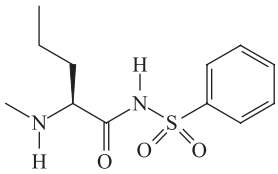
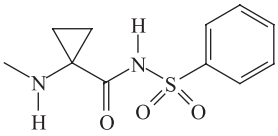
Comp.	R_1	R_2	K_i (μM), or inhibition (%)					Selectivity index (SI)			
			NS3	HLE	PPE	BPC	CatB	HLE	PPE	BPC	CatB
11	Ac		0.46[b]	0.37 ± 0.08	2% at 100 μM	30 ± 4	15 ± 2	0.8	2000[d]	70	33
12	Ac		2.3[b]	n.d.	8% at 100 μM	7.7 ± 2.4	41 ± 3	n.d.	90[d]	3.5	18
13	Ac		17	31 ± 2.5	0% at 100 μM	44 ± 4.7	0% at 100 μM	1.8	n.i.	2.6	n.i.
14	Ac		0.98[b]	0.12 ± 0.03	0% at 100 μM	60 ± 13	17 ± 3	0.12	n.i.	61	17
15	Suc		0.22[a]	19.9% at 200 μM	0% at 100 μM	18% at 200 μM	0% at 200 μM	2300[d]	n.i.	2800[d]	n.i.
16	Suc		0.091[a]	7.5% at 100 μM	0% at 100 μM	9% at 100 μM	0% at 100 μM	4200[d]	n.i.	7400[d]	n.i.
17	Suc		0.028[a]	27.5% at 200 μM	0% at 200μM	16% at 200 μM	20% at 200 μM	5800[d]	n.i.	25000[d]	17000[d]
18	Ac		0.092[a]	40% at 100 μM	0% at 100μM	0% at 100μM	0% at 100 μM	500[d]	n.i.	n.i.	n.i.
19	Ac		1.54[b]	3.8 ± 0.8	0% at 100 μM	22.8 ± 3.9	37% at 100 μM	2.5	n.i.	15	62[d]
20	Suc		0.16[a]	3.8 ± 1.2	34% at 200 μM	15% at 200 μM	0% at 100 μM	24	460[d]	4800[d]	n.i.
21	Suc		0.19[a]	49.5 ± 6.6	0% at 200 μM	21% at 200 μM	13% at 200 μM	262	n.i.	2700[d]	60[d]

Table 1 (*continued*)

Comp.	R_1	R_2	K_i (μM), or inhibition (%)					Selectivity index (SI)			
			NS3	HLE	PPE	BPC	CatB	HLE	PPE	BPC	CatB
22	Suc		0.014[a]	42% at 200 μM	9% at 200 μM	0% at 200 μM	0% at 100 μM	6300[d]	28000[d]	n.i.	n.i.
23	Suc		0.0038[a]	39.9 ± 3.7	0% at 200 μM	0% at 200 μM	0% at 200 μM	10500	n.i.	n.i.	n.i.

K_i values are presented with standard errors, based on duplicate or triplicate measurements with at least two inhibitor concentrations. Measurements of "% inhibition" at a single inhibitor concentration were performed in triplicates: n.i. = not inhibitory, n.d. = not determined.[a]Values from Ref. [10].[b]Values from Ref. [12].[c]Slow binding inhibition apparent.[d]SI values were estimated from calculated K_i values (see text).

unsaturated and saturated ketones **6**–**11** were generally effective inhibitors of HLE, BPC and cathepsin B, and thus also had low selectivity for NS3 protease.

The non-electrophilic Abu-based alcohols (**12**–**14**) and the C-terminal carboxylic acid (**19**) were effective inhibitors of HLE and BPC, and generally inhibited cathepsin B. In contrast, the compounds with a Nva, ACPC and Cys in the P1-position and a free C-terminal carboxylic acid residue (**15**–**18**) were selective inhibitors of NS3 protease, indicating that low selectivity for NS3 protease was associated with Abu in the P1-position (as in **19**). Overall, the most selective compounds were Nva- and ACPC-based acyl sulfonamides (**22** and **23**), importantly being also the most potent inhibitors of the HCV NS3 protease. The corresponding tetrazoles (**20** and **21**) showed similar selectivity, being relatively effective inhibitors only of HLE.

There was no significant difference in the selectivity between the N-terminal acetyl or succinic acid groups, as indicated by a comparison of compounds **17** and **18**.

3.3. Structure–activity relationships

All electrophilic and non-electrophilic compounds inhibited HLE to some extent. The inhibitory potency increased with the length of the aliphatic C-terminal residue (e.g. **8** was more potent than **7**) and the inhibition was affected by the structure of the P1 side chain. This was not generally the case for inhibition of NS3 protease, although it was clearly the case for the hydroxyl compounds (**13** and **14**). The structure–activity relationships for the alkyl chains were obviously different for NS3 protease and HLE. Interestingly, several compounds were equal (**1**, **8**, **11**) or better (**14**) inhibitors of HLE than of NS3 protease. In contrast, only the Nva-based pentafluoroketone (compound **1**) was a very effective inhibitor of PPE, even more potent than of NS3 protease. BPC was primarily inhibited by Abu-based compounds (**6**–**14**, **19**), while compounds with Cys, Nva or ACPC in the P1-position resulted in little or no inhibition. As for HLE, the inhibition of cathepsin B was enhanced for compounds with long aliphatic C-terminal residues, but the nature of the P1-residue was of minor importance for binding to this enzyme.

4. Discussion

The results show that the electrophilic inhibitors tested were less selective than the non-electrophilic inhibitors. Inhibition by electrophilic compounds relies on the formation of a covalent bond with the catalytic serine or cysteine residue in these proteases (Fig. 1a), corresponding to the first step of substrate hydrolysis and results in formation of a hemiketal. The primary determinant for effective inhibition is thus the electrophilicity of the compound, and resides in the R_2-moiety (Table 1) of the current inhibitors. Although many of the electrophilic compounds were potent inhibitors of HCV NS3 protease, they lack the important characteristic of selectivity, reducing their potential as drugs.

In contrast, non-electrophilic inhibitors with a C-terminal carboxylic acid (as illustrated in Fig. 1b) can be highly selective for the HCV NS3 protease, as shown herein and by Llinas-Brunet et al. [23]. However, several carboxylate groups are often not suitable in a drug molecule since they increase the tendency of the compound to be metabolized and may restrict the absorption of the drug [31]. We have previously found that both tetrazole and acyl sulfonamide groups served as bioisosteres for the C-terminal carboxylate of peptide-based HCV NS3 protease inhibitors [10]. The present study revealed that the C-terminal acyl sulfonamide compounds were highly selective for HCV NS3 protease, while the tetrazole groups were considerably less selective.

The importance of the P1-residue side chain for selectivity was also explored, using Abu, Nva and ACPC as Cys replacements. Cys could not be used as P1-residues in the electrophilic compounds due to high reactivity, but was

acceptable in the non-electrophilic compounds. Inhibitors with Cys in the P1-position (as shown in Fig. 1b) were selective for HCV NS3 protease. However, the selectivity was high even when Cys was replaced by ACPC, or Nva; the corresponding Abu compound was not selective since it was a potent inhibitor of HLE and also inhibited BPC and CatB. This is consistent with the substrate specificity of HLE, preferring P1 amino acids with medium-sized hydrophobic side chains. Although the primary specificity pocket of PPE is very similar to that of HLE, it is slightly smaller and can therefore not readily accommodate the P1-residue present in most of the inhibitors tested. PPE was therefore not inhibited to the same degree as HLE by the current compounds. BPC, in contrast, is specific for large bulky hydrophobic P1-residues. Although the inhibitors tested would thus be expected to be small enough to be accommodated by the binding pocket, the side chains would not fill out the binding pocket and the affinity is anticipated to be limited due to weak van der Waals' interactions. BPC inhibitors therefore have to incorporate other structural features, e.g. C-terminal substitutions (see above). Of all reference proteases, HLE was inhibited best by NS3 protease inhibitors and the trend in the potency of the inhibitors was often the same, which may be a reflection of the structural similarity and thus also the similar substrate specificity of the proteases. It is also a relevant enzyme since it is of human origin.

The present data also provided an additional understanding of the structure–activity relationships for these inhibitors with HCV NS3 protease. For example, our previous observation that unsaturated ketones are more potent inhibitors of NS3 protease than saturated ketones, and that both types of ketones are more potent than the corresponding alcohols [12] was not observed with the reference proteases. This demonstrates that the structure–activity relationship of saturated and unsaturated ketones is not simply correlated with the electrophilicity of the C-terminal residue, but is also an effect of specific interactions only occurring in the active site of HCV NS3 protease.

The cyclopropane group was less effective than norvaline as a P1 residue in electrophilic inhibitors for all reference proteases, although it had a minor and varying effect on the potency of non-electrophilic compounds. We have earlier observed [10] that electrophilic compounds containing the ACPC group were less efficient than compounds containing norvaline. In contrast to the reference proteases, inhibition of the HCV NS3 protease was clearly more efficient for non-electrophilic compounds with ACPC with a free C-terminal carboxylic acid phenyl acyl sulfonamide. The results indicate that the low potency of electrophilic ACPC compounds may be due to steric hindrance by the cyclopropane side chain in the formation of a covalent bond with the catalytic serine residue (Fig. 1a). The non-electrophilic compounds may not be as dependent on exact positioning of the C-terminal group relative the serine OH-group since no covalent bond is formed (Fig. 1b).

The ACPC-based compounds only inhibited HCV NS3 proteases and HLE effectively, and the inhibitory potency of the electrophilic ACPC compounds were less or equally efficient to that of the analogous non-electrophilic inhibitors (e.g. compare compounds **5** and **16**). Although the binding of α-keto acid inhibitors to HCV NS3 protease have been structurally determined [32], it appears as if the present α-keto acid inhibitor has different binding characteristics. The α-keto acid compound (compound **5**) studied here did not exhibit slow binding kinetics with HCV NS3 protease and removal of the electrophilic group did not affect inhibition. We therefore speculate that this compound does not form a covalent bond with the catalytic serine residue of the enzyme possibly due to the cyclopropyl group. The same can be speculated for the α-keto tetrazole compound (**4**), which is less potent than the non-electrophilic tetrazole (**21**). Nevertheless, the selectivity is higher for the non-electrophilic compounds even when the electrophilic analogues do not behave as expected.

5. Conclusions

The chymotrypsin-like serine protease of the bifunctional protease/helicase NS3 of HCV has long been the prime target of search for drugs against the virus. Many types of inhibitors have been developed over the last few years, but the issue of selectivity of the inhibitors for NS3 protease has generally not been addressed. Due to the large number of proteases present in the body, selectivity is an important feature of protease inhibitors designed to be used as drugs. Our data reveal important structural features of a set of different HCV NS3 protease inhibitors that correlate with inhibition of other proteases with similar structural and/or mechanistic characteristics as the HCV enzyme. In general, electrophilic compounds were less selective than non-electrophilic compounds. However, P1-residues and C-terminal groups also have a considerable effect on selectivity. The selectivity has to be experimentally evaluated for new compound classes since the effect of these different structural entities is not additive. Nevertheless, this type of information is important for guiding the design of selective drugs against HCV.

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